

# Undetectable levels of genotoxicity of SiO<sub>2</sub> nanoparticles in in vitro and in vivo tests

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**Background:** Silica dioxide (SiO<sub>2</sub>) has been used in various industrial products, including paints and coatings, plastics, synthetic rubbers, and adhesives. Several studies have investigated the genotoxic effects of SiO<sub>2</sub>; however, the results remain controversial due to variations in the evaluation methods applied in determining its physicochemical properties. Thus, well characterized chemicals and standardized methods are needed for better assessment of the genotoxicity of nanoparticles.

**Methods:** The genotoxicity of SiO<sub>2</sub> was evaluated using two types of well characterized SiO<sub>2</sub>, ie, 20 nm (–) charge (SiO<sub>2</sub><sup>EN20(–)</sup>) and 100 nm (–) charge (SiO<sub>2</sub><sup>EN100(–)</sup>). Four end point genotoxicity tests, ie, the bacterial mutation assay, in vitro chromosomal aberration test, in vivo comet assay, and in vivo micronucleus test, were conducted following the test guidelines of the Organization for Economic Cooperation and Development (OECD) with application of Good Laboratory Practice.

**Results:** No statistically significant differences were found in the bacterial mutation assay, in vitro chromosomal aberration test, in vivo comet assay, and in vivo micronucleus test when tested for induction of genotoxicity in both two types of SiO<sub>2</sub> nanoparticles.

**Conclusion:** These results suggest that SiO<sub>2</sub> nanoparticles, in particular SiO<sub>2</sub><sup>EN20(–)</sup> and SiO<sub>2</sub><sup>EN100(–)</sup>, are not genotoxic in both in vitro and in vivo systems under OECD guidelines. Further, the results were generated in accordance with OECD test guidelines, and Good Laboratory Practice application; it can be accepted as reliable information regarding SiO<sub>2</sub>-induced genotoxicity.

**Keywords:** genotoxicity test, Organization for Economic Cooperation and Development test guideline, Good Laboratory Practice, silica dioxide

## Introduction

The field of nanotechnology has been growing rapidly within various industries over the last decade. Various types of nanoparticles, including titanium oxide, zinc oxide, and silica dioxide (SiO<sub>2</sub>), are used in cosmetics, sports equipment, and building materials.<sup>1</sup> Due to their widespread applications in various industries, use of nanoparticles continues to increase. However, nanoparticles may be hazardous to human health because of their unusual physicochemical properties, eg, small size, high surface to volume ratio, chemical composition, crystallinity, electronic properties, surface structure reactivity and functional groups, inorganic or organic coatings, solubility, shape, and aggregation behavior.<sup>2–4</sup> The risk to human health arising from exposure to nanoparticles through ingestion, inhalation, and dermal absorption may increase as the applications of nanoparticles continue to increase.<sup>5–7</sup>

The Organization for Economic Cooperation and Development (OECD) has also paid attention to this problem and organized a special session in a joint meeting on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety in 2005. The OECD established the Working Party on

Manufactured Nanomaterials in 2006 for dealing with human and environmental safety issues associated with manufactured nanomaterials.<sup>8</sup>

SiO<sub>2</sub> nanoparticles have been widely used in a variety of industrial fields, including the plastics, rubber, ceramics, coatings, adhesives, and medical industries.<sup>9,10</sup> Numerous studies have reported the cytotoxicity, genotoxicity, and alterations in protein expression associated with SiO<sub>2</sub> nanoparticles in both in vitro and in vivo systems.<sup>11,12</sup> Further, it has been suggested that the genotoxicity caused by SiO<sub>2</sub> nanoparticles may be due to proinflammatory effects, modification of chromatin structure, and liberation of DNase or a potent inducer of cytogenetic damage.<sup>13,14</sup> However, these studies were controversial due to the slightly different physicochemical features involved in the synthesis, dispersion, and stability of nanoparticles in biological media.<sup>15–18</sup> Nanoparticle-related studies reported that these differences were generated by the size and surface charge of the specific nanoparticles.<sup>19–22</sup> The size of nanoparticles could influence their absorption, distribution, metabolism, and excretion.<sup>23–25</sup> The hydrodynamic size distributions of nanoparticles could be also changed by surface charge and properties, affecting agglomeration. Moreover, the surface charge of nanoparticles could affect uptake and translocation within organisms.<sup>26,27</sup> Several nanotoxicology reports have proposed that more detailed characterization of nanoparticles would yield a better understanding of their properties and interactions in vivo.<sup>28–30</sup> Therefore, the physicochemical features of nanoparticles would be the critical factor in studying nanotoxicology.

In the present study, four genotoxicity tests were conducted with well characterized SiO<sub>2</sub> nanoparticles 20 nm (SiO<sub>2</sub><sup>EN20(-)</sup>) and 100 nm (SiO<sub>2</sub><sup>EN100(-)</sup>) in size, each having a negative surface charge. In order to accurately evaluate toxicity, physicochemical characterization of nanoparticles and their behavior under physiological conditions were determined by following experimental protocols similar to those used in our previous study.<sup>31</sup> For accurate evaluation of genotoxicity, four types of in vitro and in vivo genotoxicity tests, ie, the bacterial mutation assay, in vitro chromosomal aberration test, in vivo comet assay, and in vivo micronucleus test, were performed according to OECD guidelines<sup>32–34</sup> or international validation study guideline<sup>35</sup> with the Good Laboratory Practice (GLP).

## Materials and methods

### Animal care

All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals issued by the Animal Care and Use Committee of the National Veterinary Research and Quarantine Service.

## Preparation and characterization of nanoparticles

SiO<sub>2</sub> colloids 20 nm (SiO<sub>2</sub><sup>EN20(-)</sup>) and 100 nm (SiO<sub>2</sub><sup>EN100(-)</sup>) in size were obtained from E&B Nanotech Co Ltd (Gyeonggi-do, Republic of Korea). The preparation process and physicochemical characterizations were conducted as described in a previous report.<sup>31</sup> Briefly, a JSM-6700F field emission scanning electron microscope (JOEL Ltd, Tokyo, Japan) and Zetasizer NanoZS (Malvern Instruments Ltd, Malvern, UK) were used to determine the particle size, morphology, and surface charge of SiO<sub>2</sub>. Distilled water was used as the solvent control in this study.

## Bacterial reverse mutation test

The bacterial reverse mutation test was conducted in compliance with the Korea Food and Drug Administration Notification No. 2009-116 testing guideline and OECD testing guideline 471.<sup>32</sup> The tester strains used in this study were Ames *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2uvrA in the absence and presence of metabolic activation system. All of the test strains were purchased from Molecular Toxicology Inc. (Boone, NC, USA). The metabolic activation system was prepared by mixing S9 metabolic activation (Molecular Toxicology Inc.) with Cofactor 1 from Wako Pure Chemical Industries Ltd (Osaka, Japan), giving a final concentration of 10% (volume/volume) S9. The tester strains were cultured in 2.5% nutrient broth No 2 (Oxoid Ltd, Basingstoke, UK) in a 37°C shaking incubator (120 rpm) for approximately 11 hours. The mutagenicity test was performed by mixing test substance and tester strains, which was cultured overnight in the presence and absence of the S9 mix. Next, the mixture was incubated in a water bath for 20 minutes at 37°C, mixed with top agar and a minimal amount of histidine-biotin (for *S. typhimurium* strains) or tryptophan (for *E. coli* strain), and then poured onto the surface of a gamma-ray sterile Falcon® Petri dish (Thermo Fisher Scientific, Waltham, MA, USA) containing about 15 mL of solidified bottom agar. The finished plates were incubated for 48–72 hours at 37°C. The number of revertant colonies was then counted. All plates were prepared in triplicate, and the results were tabulated as the mean ± standard deviation for each condition.

## In vitro chromosomal aberration test

The in vitro chromosomal aberration test was performed according to the Korea Food and Drug Administration Notification No. 2009-116 testing guideline and OECD testing guideline 473.<sup>33</sup> The clastogenicity of SiO<sub>2</sub> nanoparticles was evaluated for its ability to induce chromosomal aberrations

in Chinese hamster lung (CHL) fibroblast cells. A clonal subline of CHL cells was obtained from the American Type Culture Collection (Rockville, MD, USA). The karyotype of the CHL cells consisted of 25 chromosomes. The CHL cells were grown in Minimum Essential Eagle's Medium, supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin (all from Gibco BRL Life Technologies Inc., Gaithersburg, MD, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Mitomycin C (CAS No. 50-07-7; Sigma-Aldrich Co., St Louis, MO, USA) was used as a positive control, both with and without the S9 mixture. After 22 hours of incubation, colcemid was added to the cultures at a final concentration of 0.2 µg/mL, and metaphase cells were harvested by trypsinization and centrifugation. The cells were swelled by adding hypotonic (0.075 M) KCl solution for 20 minutes at 37°C, and then washed three times in ice-cold fixative (methanol to glacial acetic acid, 3:1). A few drops of cell pellet suspension were dropped onto precleaned glass microscope slides and air-dried. The slides were stained with 5% Giemsa buffer solution (Thermo Fisher Scientific, Waltham, MA, USA). The number of cells with chromosomal aberrations was recorded on 200 well spread metaphases. The classification of aberration types referred to JEMS-MMS (Japanese Environmental Mutagen Society-Mammalian Mutagenesis Study group). Aberration frequencies, defined as aberrations observed, were divided by the number of cells counted, and were analyzed using Fisher's exact test with Dunnett's adjustment, and compared with results from the solvent controls.

### In vivo alkaline comet assay

The alkaline (pH >13) comet assay was conducted according to international validation of the in vivo rodent alkaline comet assay for the detection of genotoxic carcinogens (version 14.2).<sup>35</sup> Male Crl:CD (Sprague Dawley [SD]) rats (aged 6 weeks) were used for the in vivo alkaline comet assay. The SD rats were randomized into groups containing five rats each. After a 7-day acclimation period, the test substance was administered three times by gavage at 0, 24, and 45 hours. The test doses (500, 1,000, and 2,000 mg/kg) were selected by a range-finding experiment. Following the guideline, ethyl methanesulfonate was used for the positive control at a dose of 200 mg/kg. The liver and stomach were collected from each animal and maintained in cold mincing buffer (Mg<sup>2+</sup>-free and Ca<sup>2+</sup>-free Hanks' Balanced Salt Solution (Gibco, Carlsbad, CA, USA) with 20 mM ethylenediaminetetraacetic acid (EDTA)-Na<sub>2</sub> and 10% volume/volume dimethylsulfoxide (Sigma-Aldridge, St Louis, MO, USA). The liver was minced using fine scissors

in cold mincing buffer. The stomach was opened and washed free of food using cold mincing buffer. The forestomach was discarded, and the glandular stomach was placed into cold mincing buffer and incubated on ice for 15–30 minutes. After incubation, the surface mucosa was gently scoured using a scraper. This layer was discarded subsequently, and the stomach epithelium was carefully scoured with a scraper to release the cells. The cell suspension was stored on ice for 15–30 seconds to allow large clumps to settle, and the supernatant was used to prepare the comet slides. A 10 µL aliquot of single cell suspension was mixed with 0.5% low melting agarose (Invitrogen, Carlsbad, CA, USA) and spread on comet assay slides (Travigen, Gaithersburg, MD, USA). The slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA-Na<sub>2</sub>, 10 mM Tris-base, 10% dimethylsulfoxide, 1% Triton-X [pH10]) overnight. After this incubation, the slides were placed in electrophoresis solution (0.3 M NaOH, 1 mM EDTA [pH >13]) for 20 minutes to allow for unwinding of DNA. Electrophoresis was subsequently conducted at 25 V and 300 mA for 20 minutes. The slides were then immersed in neutralization solution (0.4 M Tris-base [pH 7.5]) for at least 5 minutes and then dehydrated with absolute ethanol to fix. The cells were stained with SYBR Gold (Invitrogen) according to the manufacturer's specifications. The comet was observed via fluorescence microscopy (Nikon, Tokyo, Japan) at a magnification of 200× and analyzed by Comet Assay IV software (Perceptive Instruments Ltd, Bury St Edmunds, UK). For each sample (animal/tissue), 50 comets per slide were analyzed, with two slides scored per sample. A positive response is defined as a statistically significant change in the percent tail DNA in at least one dose group in comparison with the vehicle control value using Dunnett's test (two-sided,  $P < 0.05$ ) as well as a statistically significant linear trend test (two-sided,  $P < 0.05$ ). The positive control should produce a statistically significant increase as determined by the Student's *t*-test (one-sided,  $P < 0.025$ ).

### In vivo micronucleus test

The in vivo micronucleus test was performed in compliance with the Korea Food and Drug Administration Notification No. 2009-116 testing guideline and OECD testing guideline 474.<sup>34</sup> Out-bred 6–7-week-old mice of strain ICR were used in this study. The ICR mice were randomized into groups containing five mice each. The test substance was administered orally in three doses in volumes of 10 mL/kg. It was given twice with a 24-hour interval in between, and test subjects were sacrificed by cervical dislocation. Preparation

and staining of bone marrow was carried out according to the method described by Schmid.<sup>36</sup> In scoring the preparations, micronuclei were counted in polychromatic erythrocytes and separately in monochromatic erythrocytes. The rate of micronucleated cells, expressed as a percentage, was based on the total of polychromatic erythrocytes present in the scored optic fields. This method of scoring, which must always be followed where the test substance markedly influences the proliferation rate in bone marrow, prevented distortion of the results by the influx of peripheral blood into the damaged marrow. The scoring of micronucleated normocytes was used to recognize the presence of artifacts (which is rare in mouse preparations), which provided additional interesting information on the mode of action of the test substances. Generally, an incidence of more than one micronucleated normocyte per 1,000 polychromatic erythrocytes indicates an effect on cell stages, especially post-S-phase. The result of the statistical evaluation was deemed to be statistically significant when the *P*-value was less than 0.05. We used the Kruskal–Wallis H test and Dunnett's test for differences in numbers of micronucleated polychromatic erythrocytes between the treated and negative control groups; the Mann–Whitney *U* test for differences in numbers of micronucleated polychromatic erythrocytes between the positive and negative control groups; analysis of variance and Dunnett's test for differences in the polychromatic erythrocyte (PCE)/(PCE + normochromatic erythrocyte [NCE]) ratio between the treated and negative control groups; the Student's *t*-test for differences in the PCE/(PCE + NCE) ratio between the positive and negative control groups; and analysis of variance and Dunnett's test for comparison of animal body weight at the time of euthanasia.

## Results

### Characterization of nanoparticles

Particle size and surface charge are significant factors when determining the biological behavior of nanoparticles.<sup>31</sup> The physicochemical properties of SiO<sub>2</sub> nanoparticles were investigated in a previous study, as described briefly below.<sup>31</sup>

The average size and morphology of the SiO<sub>2</sub> nanoparticles was investigated by field emission scanning electron microscopy. The results showed a monodispersed size distribution and spherical morphology with a mean size of ~33 nm and ~90 nm for SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup>, respectively. Dynamic light scattering analysis was determined to be 23±0.1 nm and 91.6±0.5 nm for SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup>, respectively. The surface charge on the SiO<sub>2</sub> nanoparticles in distilled water suspension was determined to be negative (around -40 mV).

### Bacterial reverse mutation test

In the preliminary dose-ranging tests (data not shown), two kinds of SiO<sub>2</sub> nanoparticles, ie, SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup>, were found to have a nontoxic effect in all tester strains of *S. typhimurium* (TA98, TA100, TA1535, and TA1537) and in *E. coli* WP2*uvrA* at a dose of 5,000 µg per plate in the presence and absence of S9. Based on the data from the preliminary dose-ranging tests, we selected 5,000 µg per plate as the highest dose. As shown in Table 1, none of the tester strains showed any increase in the number of revertant colonies in comparison with the solvent control (0 µg per plate) when the bacteria were treated with the two types of SiO<sub>2</sub> nanoparticles at 313, 625, 1,250, 2,500, and 5,000 µg per plate, regardless of metabolic activation. On the other hand, the positive control group resulted in more revertant colonies than the solvent control or other SiO<sub>2</sub> treatment groups.

### In vitro chromosome aberration test

Initially, a dose-ranging test was performed to determine the test doses for use in the in vitro chromosome aberration test. The highest concentration was determined to be 1,400 µg/mL, as it showed less than 50% cytotoxicity in CHL cells (data not shown). The in vitro chromosome aberration test was conducted with SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> at concentrations of 175, 350, 700, and 1,400 µg/mL, both with and without metabolic activation (S9). Except for the positive control group, the metaphase arrested cells with structural aberrations were less than 5%. The results indicated no significant increase in SiO<sub>2</sub> nanoparticles in the treatment groups in comparison with the solvent control at the four concentrations, regardless of metabolic activation (Table 2). On the other hand, the positive control group showed significantly increased structural aberrations in comparison with the solvent control and with the other SiO<sub>2</sub> treatment groups.

### In vivo alkaline comet assay

A dose-ranging study was conducted in SD rats (data not shown), and the test doses were determined as 500, 1,000, and 2,000 mg/kg body weight. DNA damage was measured by the in vivo alkaline comet assay, following the international validation study protocol (version 14.2).<sup>35</sup> Our data showed no significant differences in percent tail DNA in single cells from the liver and stomach treated with SiO<sub>2</sub><sup>EN20(-)</sup> (Figure 1A) and SiO<sub>2</sub><sup>EN100(-)</sup> (Figure 1B). The positive control showed a significant increase in tail DNA intensity (percent tail DNA) in comparison with the negative control. Therefore, no genotoxic effect of SiO<sub>2</sub> nanoparticles (SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup>) was observed using the in vivo comet assay.



**Table 1** Numbers of revertant colonies induced by SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> in *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and *Escherichia coli* (WP2uvrA) with and without metabolic activation (S9)

Colonies per plate (mean ± standard deviation)						
Tester strain	Dose (µg per plate)	SiO <sub>2</sub> <sup>EN20(-)</sup>		SiO <sub>2</sub> <sup>EN100(-)</sup>		
		Without S9 mix	With S9 mix	Without S9 mix	With S9 mix	
TA98	0	17±6	28±4	16±2	19±6	
	313	16±4	25±6	14±2	27±3	
	625	15±5	22±7	11±2	21±5	
	1,250	17±6	22±5	10±4	21±1	
	2,500	16±3	28±7	13±5	23±2	
	5,000	11±1	18±2	16±5	20±4	
	Positive control	434±20 <sup>*a</sup>	201±26 <sup>*b</sup>	361±15 <sup>*a</sup>	199±16 <sup>*b</sup>	
	TA100	0	84±7	101±5	75±8	100±12
TA100	313	85±9	117±9	80±7	99±6	
	625	99±7	120±7	87±8	100±7	
	1,250	100±7	128±9	69±6	104±10	
	2,500	92±5	120±11	66±5	103±17	
	5,000	100±8	118±12	69±5	95±17	
	Positive control	419±46 <sup>*a</sup>	390±8 <sup>*b</sup>	361±30 <sup>*a</sup>	265±21 <sup>*b</sup>	
	TA1535	0	12±4	10±1	10±1	9±1
	TA1535	313	10±2	10±2	13±5	10±5
625		13±5	12±2	10±2	8±2	
1,250		12±2	12±5	9±2	9±3	
2,500		15±1	12±2	8±3	8±3	
5,000		18±5	14±1	9±4	10±3	
Positive control		236±26 <sup>*c</sup>	216±14 <sup>*b</sup>	323±7 <sup>*c</sup>	195±4 <sup>*b</sup>	
TA1537		0	6±2	12±2	7±1	17±3
TA1537		313	6±1	13±2	7±3	16±5
	625	4±1	15±1	7±1	14±4	
	1,250	7±0	10±1	8±3	13±4	
	2,500	6±2	8±2	8±2	15±4	
	5,000	6±0	9±1	7±2	13±3	
	Positive control	761±30 <sup>*d</sup>	184±13 <sup>*b</sup>	753±22 <sup>*d</sup>	200±13 <sup>*b</sup>	
	WP2uvrA	0	32±4	33±4	41±2	39±4
	WP2uvrA	313	35±6	44±6	39±4	39±7
625		39±6	46±9	39±5	41±6	
1,250		39±5	43±8	39±6	32±4	
2,500		37±9	46±5	42±4	36±10	
5,000		39±7	46±5	38±2	36±3	
Positive control		424±17 <sup>*a</sup>	265±8 <sup>*b</sup>	238±22 <sup>*a</sup>	242±17 <sup>*b</sup>	

**Notes:** \**P*<0.01. <sup>a</sup>2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; <sup>b</sup>2-aminoanthracene; <sup>c</sup>sodium azide; <sup>d</sup>9-aminoacridine.

**Abbreviations:** SiO<sub>2</sub><sup>EN20(-)</sup>, SiO<sub>2</sub> colloids 20 nm; SiO<sub>2</sub><sup>EN100(-)</sup>, SiO<sub>2</sub> colloids 100 nm.

## In vivo micronucleus test

In the preliminary dose-ranging test, acute oral toxicity, death, or apparent abnormality in appearance could not be determined in mice at doses ranging from 500 mg/kg to 2,000 mg/kg body weight. The PCE/(NCE + PCE) ratios were used as an index of bone marrow cytotoxicity. The ratios did not show any significant difference in the SiO<sub>2</sub> treatment groups in comparison with the solvent control group, for either size of SiO<sub>2</sub> nanoparticle. The micronucleated PCE frequencies were not statistically significant and did not yield any dose-dependent pattern among the three treatment groups in comparison with the solvent control group for either size

of SiO<sub>2</sub> nanoparticle. On the other hand, the positive control group showed a significantly increased micronucleated PCE frequency in comparison with the solvent control and SiO<sub>2</sub> treatment groups (Table 3). Therefore, both types of SiO<sub>2</sub> nanoparticles were regarded to have no genotoxic or cytotoxic potential using the current in vivo system.

## Discussion

Nanotoxicity studies have been necessary, although their results are still controversial due to variations in the physicochemical properties of the materials under investigation.<sup>19-21,37-39</sup> Further, their results could differ according to the protocol used

**Table 2** Chromosome analysis of SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> in Chinese hamster lung fibroblast cells with and without metabolic activation (S9)

Observed cell	Concentration (µg/mL)	SiO <sub>2</sub> <sup>EN20(-)</sup>		SiO <sub>2</sub> <sup>EN100(-)</sup>	
		% numerical aberration	% structural aberration (exclusive to gap)	% numerical aberration	% structural aberration (exclusive to gap)
<b>(A) 6 hours without S9 (-S9)</b>					
200	0	0.0	1.5	0.0	1.5
	175	0.5	1.0	0.0	0.0
	350	0.5	1.0	1.0	1.0
	700	0.0	0.5	0.0	1.0
	1,400	0.0	0.5	0.0	0.5
	Positive control <sup>b</sup>	0.0	54.0*	0.5	44.5*
	<b>(B) 6 hours with S9 (+S9)</b>				
200	0	0.0	1.0	0.5	0.5
	175	0.5	0.5	0.5	1.5
	350	0.0	1.5	0.5	1.0
	700	0.0	0.5	0.0	1.0
	1,400	0.0	1.0	1.0	1.5
	Positive control <sup>b</sup>	1.5	54.0*	0.5	51.5*

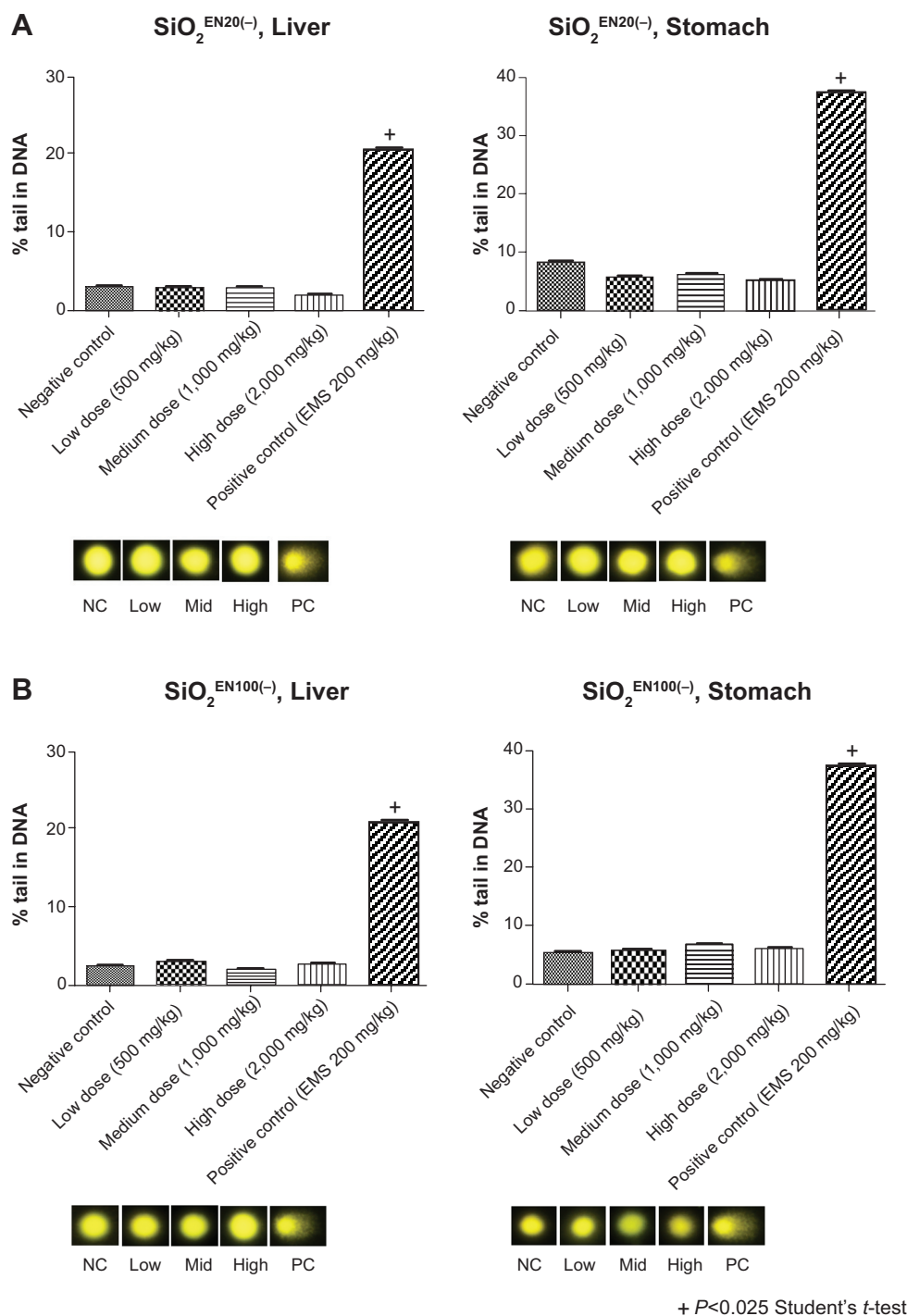
Notes: <sup>a</sup>Mitomycin C; <sup>b</sup>cyclophosphamide. \*P<0.01.

Abbreviations: SiO<sub>2</sub><sup>EN20(-)</sup>, SiO<sub>2</sub> colloids 20 nm; SiO<sub>2</sub><sup>EN100(-)</sup>, SiO<sub>2</sub> colloids 100 nm.

for evaluation. Hence, well characterized nanoparticles and approved methods are necessary when conducting nanotoxicity studies. In this study, nanogenotoxicity was investigated using well characterized SiO<sub>2</sub> nanoparticles, ie, SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup>. Characterization of these nanoparticles and their behavior was done under physiological conditions, as described in our previous study.<sup>31</sup> Next, four different end point genotoxic tests, ie, the bacterial mutation assay, in vitro chromosomal aberration test, in vivo alkaline comet assay, and in vivo micronucleus test, were conducted in accordance with OECD guidelines and an international validation study protocol using the GLP system.

The bacterial mutation assay is used to evaluate the mutagenicity of a chemical compound.<sup>1</sup> It is also reported to be an essential test within the current battery of assays required for evaluation of genotoxicity.<sup>40</sup> To date, there have been a few reports on the mutagenicity of nanoparticles such as aluminum oxide, cobalt oxide, titanium oxide, and zinc oxide based on the bacterial mutation assay. Hence, we performed this assay using strains of *S. typhimurium* TA98, TA100, TA1535, and TA1537, and *E. coli* WP2uvrA, with and without metabolic activation by S9 mixture.<sup>41-43</sup> It has recently been reported that no mutagenic potential is observed in the Ames test with and without metabolic activation.<sup>44-46</sup> In addition, Li et al investigated the genotoxic potential of exfoliated silicate nanoclay.<sup>47</sup> Our data also showed no significantly increased mutagenicity in any of the strains exposed to SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> with and without S9 (Table 1).

The in vitro chromosomal aberration test is another standard genotoxicity test, and is commonly used to observe variations in chromosomal structure induced by toxic chemicals.<sup>33</sup> Our data show that SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> did not induce any structural chromosomal aberrations in CHL cells, with or without S9 mixture (Table 2). Another previous report showed no induction of chromosomal aberrations in mammalian cells.<sup>44-46</sup> Although different types of SiO<sub>2</sub> were used in the previous studies, similar results were obtained. Hence, SiO<sub>2</sub> nanoparticles might not induce clastogenesis in mammalian cells. In vitro genotoxicity tests could afford a better understanding of their genotoxic potential by investigating various compounds. The in vivo alkaline comet assay and in vivo micronucleus test were included in the current study in order to conduct and evaluate genotoxicity tests accurately. The comet assay, a single cell gel electrophoresis assay, has been widely used for detection of DNA damage due to its simplicity, low cost, and high sensitivity.<sup>48</sup> The in vivo comet assay has been regarded as a potential replacement for the in vivo rodent hepatocyte unscheduled DNA synthesis assay, and the protocol for the in vivo comet assay has been standardized and published by an international expert group. In the present study, the in vivo comet assay was conducted using SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> and followed the standard protocol, revealing no genotoxic effect of SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> in rat liver and stomach cells (Figure 1A and 1B). Even though the majority of the studies have reported negative results for the genotoxicity of SiO<sub>2</sub>,<sup>11,17,47</sup> a few investigations have indicated otherwise.<sup>49</sup> Downs et al investigated the genotoxic



**Figure 1** DNA damage determined by in vivo comet assay in liver and stomach tissues from rats treated with (A) SiO<sub>2</sub><sup>EN20(-)</sup> and (B) SiO<sub>2</sub><sup>EN100(-)</sup>.

**Note:** The yellow and black images indicate comet images in each group.

**Abbreviations:** NC, negative control; PC, positive control; EMS, ethyl methansulfonate; SiO<sub>2</sub><sup>EN20(-)</sup>, SiO<sub>2</sub> colloids 20 nm; SiO<sub>2</sub><sup>EN100(-)</sup>, SiO<sub>2</sub> colloids 100 nm.

effects of exposure to three consecutive intravenous injections of SiO<sub>2</sub> nanoparticles by in vivo comet assay in rat liver and lung tissues, and observed the induction of DNA damage by SiO<sub>2</sub>.<sup>50</sup> Hence, the different administration route can lead to different results for genotoxicity evaluation.

The micronucleus test was used to evaluate genotoxicity by scoring the number of micronuclei under chemical

exposure. Regarding the genotoxicity induced by SiO<sub>2</sub> nanoparticles, several publications have reported on in vitro micronucleus tests.<sup>11,15,47,51,52</sup> In contrast, few in vivo micronucleus tests have been performed. Downs et al conducted in vivo micronucleus testing of SiO<sub>2</sub> nanoparticles, where they analyzed micronuclei in rat blood.<sup>50</sup> In the present study, in vivo micronucleus testing was performed with rat

**Table 3** Frequencies of MNPCE per 2,000 PCE in the bone marrow of ICR mice exposed to SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup>

Dose (mg/kg body weight)	SiO <sub>2</sub> <sup>EN20(-)</sup>		SiO <sub>2</sub> <sup>EN100(-)</sup>	
	MNPCE/2,000 PCE (mean ± SD, %)	PCE/(PCE + NCE) (mean ± SD, %)	MNPCE/2,000 PCE (mean ± SD, %)	PCE/(PCE + NCE) (mean ± SD, %)
0	0.10±0.04	53.64±2.27	0.09±0.05	53.93±2.79
500	0.13±0.07	56.11±2.46	0.06±0.02	52.98±2.76
1,000	0.10±0.06	55.11±2.81	0.14±0.09	55.19±1.55
2,000	0.10±0.05	56.02±1.32	0.09±0.04	56.24±0.89
Positive control <sup>a</sup>	4.33±0.37*	44.57±2.06*	4.82±0.92*	43.20±1.28*

Notes: <sup>a</sup>Cyclophosphamide. \*P<0.01.

Abbreviations: SD, standard deviation; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; MNPCE, micronucleated polychromatic erythrocytes; SiO<sub>2</sub><sup>EN20(-)</sup>, SiO<sub>2</sub> colloids 20 nm; SiO<sub>2</sub><sup>EN100(-)</sup>, SiO<sub>2</sub> colloids 100 nm.

bone marrow cells for the first time to determine whether SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> could induce chromosome abnormalities or mitotic apparatus in mouse model. Based on the frequencies of micronucleated PCE, no genotoxic effect of SiO<sub>2</sub> was found (Table 3). Nanotoxicology study results can be influenced by physicochemical properties, and the contrast between our results and the previous data could be due to the different administration methods used or different organs investigated, as mentioned above.

## Conclusion

In the present study, the genotoxic effects of SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> were elucidated using four genotoxicity tests, ie, the bacterial mutation assay, in vitro chromosomal aberration test, in vivo comet assay, and in vivo micronucleus test, under standardized protocols with GLP system. Although the different exposure routes from our study can induce SiO<sub>2</sub> genotoxicity in different organs in in vivo systems, our data suggest that SiO<sub>2</sub><sup>EN20(-)</sup> and SiO<sub>2</sub><sup>EN100(-)</sup> are not genotoxic substances based on the OECD test guidelines.

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## Disclosure

The authors report no conflicts of interest in this work.

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